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High throughput miniaturized screening of nanoparticle formation via inkjet printing --Manuscript Draft--

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Abstract:	The self-assembly of specific polymers into well-defined nanoparticles (NPs) is of great interest to the pharmaceutical industry as the resultant materials can act as drug delivery vehicles. In this work a high-throughput method to screen the ability of polymers to self-assemble into nanoparticles (NPs) using a picoliter ink-jet printer is presented. By dispensing polymer solutions in DMSO from the printer into the wells of a 96-well plate, containing water as an antisolvent, we screened 50 suspensions for nanoparticle formation rapidly using only nanoliter to microliter. A variety of polymer

	<p>classes were used, and in situ characterization of the sub-microliter nanosuspensions showed that the particle size distributions matched those of nanoparticles made from bulk suspensions. Dispensing organic polymer solutions into well-plates via the printer was thus shown to be a reproducible and fast method for screening nanoparticle formation which uses two to three orders of magnitude less material than conventional techniques.</p> <p>Finally, a pilot study for a high-throughput pipeline of nanoparticle production, physical property characterization and cytocompatibility demonstrated the feasibility of the printing approach for screening of nano-drug delivery formulations. Nanoparticles were produced in the well plates, characterised for size and evaluated for effects on metabolic activity of lung cancer cells.</p>
Additional Information:	
Question	Response
<p>Please submit a plain text version of your cover letter here.</p> <p>Please note, if you are submitting a revision of your manuscript, there is an opportunity for you to provide your responses to the reviewers later; please do not add them to the cover letter.</p>	<p>Dear Editor,</p> <p>Please find attached a copy of our manuscript "High throughput miniaturized screening of nanoparticles formation via inkjet printing" for your consideration regarding publication Macromolecular Materials and Engineering. The present manuscript is a revised and ameliorated version of the work previously submitted in Macromolecular Materials and Engineering encoded as mame.201700636. At the bottom of this letter, You can find a detailed, "point-by-point", answers report to the points previously raised by the Reviewers. All the new/alterd sections are reported in red or underlined in red.</p> <p>This manuscript describes a new high throughput approach to screen the ability of polymeric materials to self-assemble into nanoparticles (NPs) via inkjet printing. The current established nanoprecipitation method, is relatively simple but requires mg of polymers and one day for the complete removal of the organic solvent before size analysis can be undertaken. Employing more sophisticated technologies to test the ability of new materials to form nanoparticles in an easy, fast and automated way that will enable a rapid insight on the properties of the resulting NPs, which is still an outstanding challenge in this field.</p> <p>In order to address some of the current limitations in the screening of self-assembling polymers, we propose a new high-throughput, reproducible and fully automated method to screen different polymers via a 2-D pico-litre capable ink-jet printer dispensing μl of the organic polymer-containing solvent into an anti-solvent (water) contained in a 96-well plate system. It is worth mentioning that the polymers used in this work demonstrate different and heterogenous structures (linear block copolymers, random copolymers and grafted copolymers), with well-known assembling properties in water at the concentrations explored during the development of the method. For a final NP concentration of 500 $\mu\text{g/ml}$ only 100 μg of polymer in 10 μl of DMSO were dispensed which resulted 2 to 3 orders of magnitude less material than conventional state-of-the-art techniques. Dispensing took just 10s per sample and also displayed the advantage of easily storing all the prepared samples in 96-wellplates. The methodology adopted in this work proved to be highly reproducible, and reliable in distinguishing between materials able to self-assemble in water from water-soluble polymers unable to produce NPs.</p> <p>The experimental core of the manuscript has been divided into two subsections. The first set of results have been exploited to validate the 2D-printing methodology by means of two different analytical assays such as DLS and TEM. The second experimental section presents a pilot study scenario of high throughput pipeline of nanoparticle production, characterization and cell assays. This proof-of-concept experiment enabled the printing approach for screening of nano-drug delivery formulations in a high throughput fashion. The above methodology not only addresses the problem of miniaturization but also offers wide applicability as a routine tool for the screening and analysis of self-assembling materials, a necessity in both formulation and drug delivery fields, and should be easily adaptable from a lab setting to an industrial platform.</p> <p>For the above-mentioned reasons, we believe that this method paper can be of significant interest in the understanding of the self-assembling molecules/macromolecules and micro-to-nanomaterials applications and can be taken under consideration for publication in Macromolecular Materials and Engineering. In</p>

addition. from the previous version, new co-authors, namely Dr Amanda Pearce, Dr Robert J. Cavanagh, Dr Marion J. Limo and Dipak Gordhan, have been added in order to cover new expertise needed to refine and enhance the quality of the final work. I confirm that all authors have seen and approved the manuscript and that there are no conflicts of interest concerning possible publication. Thank you in advance for your consideration of the manuscript.

Yours sincerely,
Cameron Alexander, Jonathan Burley and Vincenzo Taresco

Reviewers comments from previous version (mame.201700636)

Reviewer #1: The authors report on the fabrication of self-assembled particles based on the co-solvent method employed for amphiphilic block copolymers. The main Topic is related to the use of inkjet printer-based Addition of polymer slutions in a good solvent to water as selective solvent. The authors present their results mainly as novel with regards to the inkjet method, firstly introduced by Hauschild et al. In my opinion, the presented paper is of minor novelty. The Basic principle of particle formation by the described addition of a polymer solution exactly parallels the common batch preparation. The only difference is the smaller solution volume. It does not become clear, how this influence the size, shape (or even more important) the size distribution (in comparison to state-of-the-art batch experiments). The potentially interesting input of a limited amount of sample on the structure formation process at the injection spot is extinguished by the multiple droplet injection as described by the authors. In Addition, I do not understand the idea of a high throughput sample preparation without immediate or at least very fast Analysis (in contrast to the batch DLS and TEM measurements provided here). Also, the enormous discrepancy of sizes determined by DLS and TEM (nearly a factor of 2 in size) has only be described by the authors as an Observation, by no explanation was given. In addition, the "raw" data provided from the DLS measurements nearly all show an irritating LOWER value of the correlation function at very small correlation times compared to slightly larger (typically below 1 micro second), which is physical nonsense.

All in all, I do not recommend publication of the manuscript in its present form.

We pondered the comments of the Reviewer and as consequences we have deeply reconsidered and ameliorated part of the high throughput experimental work. We exploited the previously reported set of experiments to validate the use of the ink-jet printer in materials self-assembling screening. Subsequently, to address the perplexity of the Reviewer we added a second fully high throughput session with a completely new set of experiments, spanning from fast DLS scanning to fast cell viability assay. In particular, the newly introduced experimental section presents a pilot study scenario of high throughput pipeline of nanoparticle production, characterization and cell assays. This proof-of-concept experiment enabled the printing approach for screening of nano-drug delivery formulations in a high throughput fashion. We also gave a different explanation of the TEM and DLS sessions.

Reviewer #3: The work describes an exploration of inkjet printing as a means of high throughput screening for nanoparticle self-assembly using 5 different polymers in DMSO solutions, printed into water as an anti-solvent. I think more details are required to be considered for publishing in this journal, as I have suggested below. As mentioned at the beginning, I think with additional results this will be a very good publication.

1. The molecular weights of the different polymers are not included in the manuscript, that I can see. This is a very important parameter to include in terms of inkjet printing as it is known that long chain length polymers undergo scission during printing.
5. Can the author include a comment if all polymers are expected to be amorphous?
1-5. Following the Reviewer suggestions, we addressed some additional points adding a new table (now Table 1) presenting polymer properties. The table has been introduced and described in the main manuscript backbone.

2. I feel that the inkjet printing section needs to be explored in more detail. There are very limited results regarding the definition of drop volume and drop shape. Often there is rapid precipitation of polymer at a liquid-liquid interface and so the drop ejected from the printer may be of great importance. Drop volumes and errors are very important if that is the case. This can be carried out through printing approximately 100k - 500k drops and measuring the mass and converting to volume. Alternatively, a good drop watcher system can be employed.

There are no details about the voltage, waveform and drop shape. When using printing it is important to know that the drops are being ejected as single drops or if they have satellites associated with the main drop. This is often carried out during the experimental development but is very important to include in the report. Can the author include information about the change in behaviour with drop size, temperature, etc.?

2. A fully and detailed description of the inkjet printing section has been added to the manuscript in the method section, emphasising the droplet volume behaviour and the lack of satellites.

	<p>3. It is hard to assess without the original files but the TEM images provided are not sufficient for accurate particle size measurement in my opinion and should not be used as a comparison with the DLS. Higher magnification is likely possible or may already be available and should be included instead.</p> <p>3. An ImageJ analysis of the TEM pictures were reported and added to the main backbone of the manuscript. As the Reviewer highlighted is not possible to compare the two techniques in terms of absolute size values. We wanted to corroborate the formation of Nanoparticles also via an orthogonal “dry” technique. It is not our aim to study a possible variation in shapes or behaviour via TEM at this stage. For the reasons mentioned above, we moved the TEM session in the SI.</p> <p>4. Table 1 shows that there are significant statistical differences for some of the results (manual compared with printing). It would be good to note this instead of the phrase "almost perfect agreement". Can the authors comment on the level of control needed for this to be a successful technique? Maybe 40nm difference in radius is not important but it would be good to comment as this is a significant percentage of the overall size.</p> <p>4. We agreed with the Reviewer comments that 40 nm might be consistent as sizes difference but at this stage of the work we are focusing in assessing whether the materials are able or not to self-assemble and at the late stage to evaluate whether there is a trend with the traditional methodology. As it is possible to notice a similar trend in size variation from the two NPs preparation techniques. Thus, the size distribution table (now table 2) has been described accordingly.</p>
Do you or any of your co-authors have a conflict of interest to declare?	No. The authors declare no conflict of interest.

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High throughput miniaturized screening of nanoparticle formation via inkjet printing

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Keywords: self-assembling; ink-jet printer; high throughput-miniaturized screening; nanoparticles

Abstract

The self-assembly of specific polymers into well-defined nanoparticles (NPs) is of great interest to the pharmaceutical industry as the resultant materials can act as drug delivery vehicles. In this work a high-throughput method to screen the ability of polymers to self-assemble into nanoparticles (NPs) using a picoliter ink-jet printer is presented. By dispensing polymer solutions in DMSO from the printer into the wells of a 96-well plate, containing water as an

antisolvent, we screened 50 suspensions for nanoparticle formation rapidly using only nanoliter to microliter. A variety of polymer classes were used, and in situ characterization of the sub-microliter nanosuspensions showed that the particle size distributions matched those of nanoparticles made from bulk suspensions. Dispensing organic polymer solutions into well-plates via the printer was thus shown to be a reproducible and fast method for screening nanoparticle formation which uses two to three orders of magnitude less material than conventional techniques.

Finally, a pilot study for a high-throughput pipeline of nanoparticle production, physical property characterization and cytocompatibility demonstrated the feasibility of the printing approach for screening of nano-drug delivery formulations. Nanoparticles were produced in the well plates, characterised for size and evaluated for effects on metabolic activity of lung cancer cells.

1. Introduction

Over the last few decades, the application of nanotechnologies to the biomedical and pharmaceutical field has significantly enhanced global health, improving diagnosis and treatment of several diseases.^{[1],[2]} A plethora of different materials has been used to produce nanosized carriers, from biodegradable polymers and lipids up to inorganic materials, and to date 51 nanoparticle (NP) formulations have been approved by the FDA for clinical applications (e.g. *Neulasta*, *Copaxone*^[3]). However, NP formulations can be challenging as there is a constant need to establish both functional and novel chemical and technological approaches in order to i) optimize the self-assembly behaviour of the base materials into NPs; ii) control and establish the reproducibility of the size, shape and colloidal behaviour of the NPs in accordance to the needs of the biomedical application; iii) modulate the encapsulation and the release rate of well-defined drugs and iv) minimise the use of surfactants and organic solvents in the preparation process. To address some of these needs, much interest has been focused on

amphiphilic block copolymers due to their versatility, their tunable properties and their ability to self-assemble into various types of NPs (micelles, nanospheres, nanocapsules and polymersomes).^{[4],[5],[6]} A range of different preparation techniques can be used, with typical polymeric NP preparation techniques involving in situ precipitation and solvent displacement, where the polymeric NPs self-assemble in the presence of a solvent (usually water), which is unfavourable for one block of the amphiphilic polymer. Such methods reduce the need of surfactants in the formulation process and utilise water-miscible solvents that can be easily removed if required.

Nevertheless, conventional methods need materials in the milligram scale, require days for the full removal of the solvent, face issues with scalability and batch-to-batch variability and frequently result in NPs with wide size distributions. Employing more sophisticated technologies to test new materials in an easy, fast and automated way that will enable the modulation of the NPs properties is still an unresolved challenge in this field. In this context, there is a real need for high throughput technologies to screen rapidly a large number of materials and optimise their formulation conditions. For example, the use of inkjet technology to obtain polymeric microspheres with defined sizes,^[7] protein encapsulated polymeric microstructures^[8] and loaded drug-polymer micro-particles^[9] has been well established in the literature. On this basis, inkjet printing could potentially be a promising alternative to the conventional methods used for the production of NPs. Inkjet printing is a versatile, scalable and relatively inexpensive method of depositing small volumes of solutions, even down to the picoliter range, with remarkable accuracy and reproducibility. As inkjet printers become more commercially available, their use in the field of drug delivery has increased.

Different materials have been printed by inkjet systems spanning from cells,^[10] to genes^{[11],[12]} or proteins^[13] to polymers^[14] to nanomaterials and some pharmaceutical formulations.^{[15],[16],[17]}

In a singular and pioneering work by Hauschild *et. al.*,^[15] unilamellar nano-vesicles were printed from a conventional desktop inkjet printer, using ethanol solutions of both lipid-like and

1 amphiphilic copolymers, resulting in NPs with reproducible sizes ranging between 50 and 220
2 nm. In the same work, it was also shown that fluorescein loaded vesicles with narrow-size
3 distributions could be directly produced via the printing method.^[15] However, in the literature,
4 preparation of these NPs with the use of a conventional desk-top inkjet printer required solution
5 volumes at the scale of 1 mL, similar to the ones needed for conventional individual batches
6 prepared manually. While Hauschild *et.al* suggested that the inkjet NP-fabrication method
7 would be relatively easy to integrate within a high-throughput platform, no reports to date exist
8 in which ink-jet printing has been adopted to rapidly screen polymers able to self-assemble in
9 water by using nano-to-micrograms of materials.

10 Therefore, in the present work we show a high-throughput, fast, reproducible and automated
11 method to screen the self-assembling properties of different polymers into nanoparticles. The
12 screening is accomplished via a 2-D picolitre-capable ink-jet printer dispensing polymer
13 solutions into an anti-solvent (water) by using few microgram of final materials, contained in a
14 96-well plate system. We have employed a wide range of readily available commercial
15 polymers, as well as some customised polymers from our own laboratories. Polymers with
16 different architectures, linear block copolymers and grafted copolymers (polymer properties
17 described in **Error! Reference source not found.**), were screened by using only micro-
18 amounts/volumes (for a final NP concentration of 500 µg/mL only 100 µg of polymer was
19 required in 10 µL of DMSO dispensed in around 10 s in 200 µL of water) to evaluate their
20 ability to self-assemble and to measure their size in different concentrations. All the
21 concentrations were chosen based on previously published methods shown to allow polymer
22 chains to self-assemble in water, and the data for these polymers were compared against
23 appropriate control materials.^{[18],[19]}

24 The versatility of the ink-jet printing technology presented here as a miniaturized screening
25 method may have implications for multiple pharmaceuticals platforms.

1 In this regards, Giardiello *et al.*^[20] developed an accelerated nanomedicine platform to generate
2 a potential aqueous paediatric HIV nanotherapy, targeting oral dose, with clinical translation
3 and regulatory approval for human evaluation. Giardiello's small-scale screening used 1 mg of
4 drug compound, generating large libraries of solid drug nanoparticles (160 individual
5 components), and iterative pharmacological and chemical evaluation established potential
6 candidates for progression through to clinical manufacture. The inkjet NP-fabrication method
7 of the present work might be employed as a miniaturized pre-formulation screening step to be
8 integrated within the work of Giardiello reported above or within general accelerated
9 nanomedicine platform approaches where nanodispersions are screened as a valuable
10 alternative to molecular solutions.^[21]

11 Finally, as a case study for a high throughput pipeline for nanoparticle production,
12 characterization and effects on cell metabolic activity, a small experiment with selected
13 polymers was performed. This proof-of-concept 'on-line assay' demonstrated the feasibility of
14 the printing approach to screen formulations, from nano-particle preparation to preliminary
15 cytotoxicity assays in a high-throughput fashion.

2. Results and Discussion

2.1 Validation of the use of ink-jet printing as a miniaturized methodology to screen polymers self-assembling in water by means of traditional analysis.

2.1.1 Known block-copolymer structures (*mPEG-b-PCL*, *mPEG-b-PLGA* and *mPEG-b-PeDL*)

Block co-polymers widely used in the drug delivery field such as *mPEG-b-PCL* and *mPEG-b-PLGA*,^{[22],[23],[24]} well-known to self-assemble in water into NPs, were first used to test the usefulness of the ink-jet printer as a NP formation screening technique. *mPEG-b-PCL* and *mPEG-b-PLGA* (50/50) were initially analysed at one single concentration (**Error! Reference source not found.a**). Subsequently, to evaluate the reproducibility of the printer, replicates of *mPEG-b-PLGA* (50/50) were dispensed at different concentrations (**Error! Reference source not found.-c** and SI). Moreover, a third block copolymer, *mPEG-b-PeDL*, with the hydrophobic part derived from renewable sources,^[25,26] thus, a convenient “green” replacement for PCL, was dispensed at two different concentrations in duplicate during the first set of experiments. Consistent reproducibility of *mPEG-b-PLGA* samples in terms of size distribution was observed and a similar trend with the common individual manual NP sample preparation method was also observed (**SI, Tables 1 and 2** for the reproducibility experiments).

Contrary to some typical nanoparticle preparation protocols, where the nanoparticle suspension is filtered for the removal of any aggregates, we did not filter the solutions containing the fabricated nanoparticles prior to the analysis, due to the small amount of suspension produced in the 96-well plate system. Thus, at the stage of the light scattering measurements, formation of precipitates during the measurements were noted (see correlograms and intensity distributions in SI, **Error! Reference source not found.** and **Error! Reference source not found.**). Further manual/conventional experiments were performed, neglecting the filtration step, in order to investigate the ability of our method to mimic any events occurring at the larger

mg scale. Consistently, aggregates were observed also in this latter conventional way to produce NPs (**Error! Reference source not found.d**). In order to probe better these common outcomes, a comparison with literature of similar polymeric structures in terms of chemistry and NP formation technique was established. In previously published work,^{[19],[27]} the ability of mPEG-*b*-PCL and PEG-*b*-PεDL to self-assemble and produce micelles was tested by dissolving the copolymers in acetone and adding the organic solutions at a fixed rate into HPLC grade water by using a pump system (each set of experiments involved 50 mg of polymer). Once the acetone was removed by slow evaporation under constant stirring, the micellar nano-suspensions were subsequently filtered through a membrane syringe filter (pore size: 220 nm) (Millex-LG, Millipore Co., USA) in order to remove aggregates and impurities formed during the self-assembling mechanism.^{[19],[28],[29]} We found possible formation of aggregates of similar materials by the printed method as well, where just a few µg were used instead of the 50 mg used in the existing literature.

2.1.2 PGA and its derivatives tested and compared to manual experiments

Further investigations were performed using Poly(glycerol-adipate) (PGA)^{[30],[31]} and some of its modifications (**Error! Reference source not found.a**) as a polymeric platform. PGA is an amphiphilic polymer, which can be enzymatically synthesised from divinyl adipate and glycerol, with a tunable amount of branches along the main backbone.^[30] Due to its intrinsically non-toxic amphiphilic nature, this kind of polyester has been further functionalized to engineer new polymeric platforms for nanotechnology in the healthcare field. In particular, it has been further modified with biological molecules such as fatty acids and amino acids in order to enhance drug protein or nucleic acids interactions or encapsulation, or simply to tailor its hydrophobic-hydrophilic balance.^{[30],[31],[32]} PGA derivatives, including the bare polymer,

showed molecular weights in the order of 20000 Da and all the materials were found to present an amorphous behaviour with T_g transitions ranging from -33 to 49 °C (see also **Error! Reference source not found.**)^[18] Full characterisation of these polymers has been published in the past^[18] and for this reason is not included in this present work.

Unmodified and amino-acid modified (N-acylated Phenylalanine and N-acylated Tryptophan) PGAs, dispensed in various concentrations, formed well defined nanoparticles with hydrodynamic diameters in the range of 50 nm to 150 nm (**Error! Reference source not found.b**). The diameter of the particles fabricated by the printer showed a similar trend to those prepared by the traditional manual method (**Error! Reference source not found.**) although some variance in final size values can be appreciated. These alterations may be attributed to several variables such as the lack of stirring in the printing step, the different rate of addition of the polymeric solution in water and the relative kinetics of solvent mixing. All the modified PGA adopted in the present work had 50% mol/mol average ratio of functionalisation. PGA-Phe and PGA-Trp were also re-printed thrice to estimate the reproducibility of the methodology with these grafted polymers. As can be seen from **Error! Reference source not found.** and **Error! Reference source not found.** both sizes and PDIs were consistent by the DLS measurements.

In the attempt to assess the shape of some printed NPs in the dry state, we used TEM imaging. DLS allows the evaluation of the solvated NPs' size, where the values reported correspond to the NPs hydrodynamic diameter and as such are affected by solvent molecules associated to the nanomaterials, while TEM facilitates measurement of the dry and thus more compacted state of the nanoparticles.

PGA Trp (54±11 nm, **Error! Reference source not found.a**) and PGA Phe NPs (77±18 nm, **Error! Reference source not found.b**) showed a distribution of circular shaped NPs by TEM. As expected the dry diameter sizes are different from the ones recorded by DLS (PGA Trp 64±1 nm and PGA Phe 133±1 nm). **It can also be seen that some of the particles do not show a well-**

defined spherical shape. We speculate that this effect is due to the partial evaporation of DMSO from the TEM specimen due to lack of further treatment, such as dialysis, as in common NPs formulation approaches. Nevertheless, TEM pictures showed a similar trend of DLS in final particles sizes behaviour, where PGA Trp NPs were smaller than PGA Phe NPs and provided a promising first insight into the adoption of the printing methodology as a screening technique for pre-formulation steps. These size differences between these two techniques are large but consistent and it is a phenomenon that is well established and reported.^[33]

2.1.3 Large scale batch experiments

To further validate the printing screening approach, a larger scale batch experiment was performed. PGA was employed as a model system in the fabrication of 60 samples at two different concentrations (50 and 100 µg/mL) in one single printing event that required less than 20 minutes to be completed. All the samples were contained in a single well-plate and sizes were measured directly in a DLS plate-reader. It can be observed from **SI-Figure 17** that the printing batch-screening showed a good reproducibility in terms of sizes and particle distributions at the two explored concentrations. Interestingly, despite the similarity in concentration values, it is possible to evaluate differences in sizes with a marginal deviation between the two values, 71 ± 4 nm for the 100 µg/mL and 65 ± 2 for the 50 µg/mL. This additional experiment may confirm the use of a printer to direct the self-assembling of amphiphilic materials in a large scale, such as for industrial batch-screening.

2.1.4 Negative printing controls and validation of method

Negative “printing” controls were also performed using polymers that were not expected to self-assemble once dispensed in water without the use of a second surfactant,^{[34],[35]} PVP and PVPVA demonstrated very poor correlation on the DLS (**Error! Reference source not found.**). These latter sets of observations highlight the possibility to distinguish between materials able to self-assemble in water and those which do not self-assemble, thus avoiding any “false-positive” results.

2.2 Case study scenario: NPs production, self-assembling screening and biological response of selected polymeric materials in high-throughput fashion.

A pilot study investigating a high throughput pipeline of printed nanoparticle production, characterization and cell-based screening was performed. The above experiments were repeated on a small scale in an entirely high-throughput fashion, with the intention that this process may be translated as an example to validate the use of polymers as a carrier for drug delivery formulations. Five samples were chosen for the study: three PGA variants (namely PGA, PGA Phe and PGA Trp) as well as two negative control polymers (PVP and PVPVA) that previously were found not to self-assemble. All the materials were printed in a well-plate containing water at two different concentrations (50 and 250 µg/mL) and were directly analysed for their self-assembling ability by a high throughput DLS plate reader (HT-DLS). To corroborate the concept of pipeline screening a high throughput cell cytotoxicity assay (MTS) was carried out directly on the printed samples without any further or intermediate purifications.

The HT-DLS data of the printed versus the samples prepared in bulk revealed a similarity in the sizes obtained for all the PGA variant polymers was noticed (**Error! Reference source not found.** see also **SI-Figure 18**). Two trends were observed for both the NP preparation techniques adopted. Both PGA-Phe and PGA-Trp at the two explored concentrations showed

sizes bigger than pure PGA NPs. At the same time, a clear enlargement of NPs sizes for all the explored materials was observed on increasing the concentration from 50 $\mu\text{g/mL}$ to 250 $\mu\text{g/mL}$. Cytotoxicity is of critical importance for the clinical translation of any kind of nanosized carrier. The formulation strategies employed for NPs production, including the presence of surfactants and of course of solvent traces, significantly affect cell metabolism. In our formulations, the concentration of DMSO in the final nanosuspensions was determined to be non-cytotoxic (**Error! Reference source not found.**), in agreement with previous evidence.^[36] In fact, as evident in **Error! Reference source not found.**, the different NP formulations obtained by printing, did not show any negative effects on the metabolic activity of H1299 lung cancer cells after 24h of treatment, in line with the data reported for NPs made by similar polymers but through different formulation processes.^[36] Therefore, the high throughput method of directly transferring the formulations in the well plates to any cell-based assays required, including cytotoxicity assays, has been validated. It should be noted that previous literature has shown that PGA and its derivatives are not toxic (although different cell lines have been used in previous works).^{[18],[31],[37]}

Taking this into consideration, the successful *in vitro* application of different polymers reported throughout the present manuscript was validated. The polymers selected had different structures, molecular weight distributions and amphiphilic properties. Both size measurement distributions and repeat sequences of negative controls showed an excellent match with manually-prepared nanosuspensions. At the same time, the precision of the system allowed the fast and accurate calculation of the number of droplets needed to achieve the final polymer concentrations. The suitability and versatility of the technology presented here as a miniaturized screening method may have implications for multiple pharma and medical platforms.

3. Conclusions

The results presented support the adoption of inkjet printing as a high throughput NP formulation investigation method, with the key strengths of the method lying in the automation, low amounts used, and reproducibility. To probe the self-assembling behaviour of any of the materials tested in the present work at the final concentration of 1 mg/mL (in triplicate), a total of 600 µg of polymer was required. This could be dispensed in a few minutes without any human intervention (see Calculation sheet). The rapid addition of small amounts of polymer-DMSO solution allowed the quick diffusion of the DMSO without the need for mechanical stirring and thus the controlled formation of the NPs.

Known block copolymers such as mPEG-*b*-PCL and mPEG-*b*-PLGA were initially used, as models, to explore the ability of an ink-jet printer to produce NPs inside a well-plate. The printed NPs showed good reproducibility throughout the sample polymer set and a close match with the particles prepared manually. Interestingly, although a limited amount of material was used, it was possible to link aggregate formation with the mg scale samples formed by the conventional method, which is consistent with evidence of aggregation previously reported in literature. By adopting the same nozzle/instrument conformation it is also possible to use solvents such as water, DMF, DMSO and mixtures with low boiling point solvents. However, for the scope of screening the self-assembling of materials mimicking a nanoprecipitation process, there are two key advantages in the selection of DMSO as solvent. DMSO is water miscible, allowing the nanoparticle formation via solvent displacement to take place, and DMSO has a high boiling point to avoid nozzle clogging. DMSO is also the most common solvent used in the drug discovery field to dissolve, screen and store the thousands of compounds synthesized and designed by combinatorial chemistry.^[38] Additionally, the adoption of the ink-jet printer to probe the ability of polymer chains to self-assemble in NPs showed remarkable advantages, including the use of limited amounts of materials, full

automation, low amount of waste, reduction of toxic solvents, fast screening and miniaturized storage. In fact, taking into account this latter variable, all the printed samples explored in the present work (over 100, considering all the replicates) were stored in a couple of 96 well-plates while the same samples prepared manually needed to be stored in as many vials as number of wells to match the full comparison. Not only, the reduction of storage space renders this technique extremely appealing in terms of easy allocation and sample/data retrieval. Even on the repeat printing sequences of the negative controls (PVP and PVPVA) the results were completely reproducible.

Finally, a pilot study of high throughput pipeline of nanoparticle production, characterization and cell assay demonstrated the feasibility of the printing approach for screening of nanodrug delivery formulations. We believe the high throughput self-assembly characterisation via 2D inkjet printing has the potential to become a standard method for particle engineering and rapid formulation development.

4. Experimental Section

4.1 Printing set-up and work flow conditions

Prior to dispensing the DMSO polymer solution into a 96-well plate filled with antisolvent (water, 200 μ L per well), the target had to be programmatically defined. Firstly, the outer dimensions of the plate were added to the software sciFLEXARRAYER (Scienion AG, version 2.09.002) followed by defining the number of wells, well distance, well depth and the spot area (area within the target designated for spotting). Within the spot area, a field pattern was defined as a 9x9 spot matrix. After setting up the field pattern, the field setup was used to address the wells from the probe substrate to the spots. The probe substrate consists of the well plate containing the polymer solution with a starting concentration of 10 mg/mL (**Error! Reference source not found.**(A)). DMSO polymer solutions were dispensed via a piezo electric inkjet printer (Sciflexarray S5, Scienion) using a 90 μ m orifice nozzle. **The nozzle was programmed**

to dispense the DMSO polymer solutions into the well from a vertical distance of *circa* 10-20 mm from the well-plate, without touching the water surface. In all experiments, 25 spots in the defined field (Scheme 1 B, highlighted in light green) were selected as the “print pattern”. The droplet size was controlled by the values of the voltage and electrical pulse. The voltage and electrical pulse were also tuned to prevent the occurrence of satellite droplets (see Support Info, **Error! Reference source not found., Error! Reference source not found.**). Images of the drop formation and droplet size were obtained using the printer software. The final spots were imaged using the Leica MZ16 stereomicroscope. Depending on the concentration to be reached, the number of drops per spot was adjusted accordingly; for example, 1000 drops (each drop shows a volume in between 360 and 400 pL dispensed every 30 μ s) per spot for 2.0 mg/mL and 3 drops per spot for 0.001 mg/mL. The number of drops per spot were selected in such a way that the volume aspired by the nozzle (max 10 μ L) in the beginning of a run was sufficient to dispense the whole targeted final concentration of nanoparticles in the water volume in the well. Therefore, a multitude of runs were printed into the same well in order to achieve the desired polymer concentration. The amount of polymeric material used in the experiments is one of the strongest points of the method. In a routine experiment DMSO solution (10 mg/mL) droplets with nominal volumes ranging from 360 pL to 400 pL were dispensed into the targeted well filled with water, at a 300 Hz jetting frequency by adjusting the voltage and pulse between 98 V to 109 V and 53 μ s to 65 μ s, respectively (see Support Info). The nozzle was washed with DMF, in between each printing cycle, as part of the automated printing/washing loop. Once DMSO solutions were printed, the fast diffusion of the organic solvent into water drove the self-assembling of the polymers. DMSO was chosen both due to its high evaporation point that avoids clogging of the printer nozzle^[39], because it is water miscible and also because it acts as a common solvent for many drugs and polymers^{[40],[38]}. For the investigation of the NP formation at the highest polymer concentration of 2 mg/mL, the amount of polymer needed for

the printing was 400 μg , while in a conventional nanoprecipitation this is typically up to 10 mg or more.

4.2 Traditional methodology: manual preparation

A subset of nanoparticle suspensions were prepared following a protocol previously established^[33] (final NPs concentration equal to 2 mg/mL): 2 mL of a stock solution of polymer in organic solvent (DMSO, with the exception of mPEG-*b*-PCL where a mixture of DMSO:Acetone in 50:50 % vol/vol was adopted) were added dropwise to 5 mL of filtered water (HPLC-grade). The solution was left overnight under constant gentle stirring for the evaporation of the organic solvent, when the final nanosuspension was collected for size analysis via DLS. This procedure required approximately 10 mg of polymer for each final suspension, 2 mL of DMSO, 5 mL of water and lasted approximately 24 hours. Every single NPs suspension needed to be stored in a vial. In comparison, the same experiment performed by ink-jet printing required 0.4 mg of polymer, 40 μL of DMSO and 200 μL of water. This latter sample was confined to one single well of a 96-well plate.

4.3 Nanoparticle Size Analysis

Dynamic Light Scattering (DLS) measurements were conducted in triplicate on the final unfiltered nanosuspensions, produced both manually and printed, using a Malvern Zetasizer Nano ZS at 25°C (scattering angle 173°, laser of 633 nm). TEM samples were prepared as follows; the sample in aqueous suspension (13 μL) was added to a copper grid (Formvar/Carbon film 200 mesh Copper (100)). The sample was left on the grid for 10 minutes and then the excess was removed using filter paper. Then, freshly prepared uranyl acetate (2%, 13 μL) was added on the grid and was left for 5 minutes before the removal of the excess with filter paper. The grid was allowed to dry under a fume hood for a minimum of 30 minutes prior to use. TEM images were captured using the FEI Biotwin-12 TEM equipped with a digital camera at the

Nanoscale and Microscale Research Centre (NMRC) of the University of Nottingham. TEM image analysis for the size distribution of the NPs was performed via ImageJ (version 1.51j8).^[41]

4.3.1 Particle Size Characterization using High Throughput Dynamic Light Scattering (HT-DLS)

Particle size analysis of the final unfiltered nanosuspensions, produced both manually and printed was performed using a Wyatt DynaPro Plate Reader II DLS instrument which has a laser wavelength of 817.28 nm and a scattering angle of 158°. Experimental temperature was set to 25 °C and auto attenuation was enabled to determine the optimal laser power and attenuation. For a measurement in each well containing 100 µL of sample, 10 acquisitions were carried out, each for 10 seconds. DYNAMICS software implementing the Dynals algorithm was used for the data analysis.

4.4 Metabolic activity

H1299 lung cancer cells were obtained from the American Type Culture Collection, cultured at 37°C in a humidified atmosphere containing 5% CO₂ and grown routinely in RPMI supplemented with 10% FBS, 100 unit/mL penicillin and 100 µg/mL streptomycin. H1299 cells were seeded at a density of 2x10⁴ cells/well in 96 well plates and cultured in 100 µl RPMI medium containing 10% FBS for 24h prior to NP treatment. NP suspensions were applied in culture medium at a final concentration of 125 µg/ml for 24h.

Additionally, to study the cytocompatibility of the DMSO concentration present in the final NP dispersions, medium containing 0.75% (v/v) DMSO was applied to cells. Cells were also treated with 0.1% (v/v) Triton-X 100 and culture medium alone for 24h for use as positive and negative controls, respectively. Following treatment, cells were washed with PBS and incubated with 20 µl MTS solution (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega)

diluted with 100 μ l medium per well for 3h. Absorbance was then read at 490 nm in a microplate reader (Tecan Spark 10M, UK). Relative metabolic activity was calculated with the absorbance at 490 nm for the negative control set as 100%, and the positive control as 0%.

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Data access statement

All raw data created during this research are openly available from the corresponding author (vincenzo.taresco@nottingham.ac.uk) and at the University of Nottingham Research Data Management Repository (<https://rdmc.nottingham.ac.uk/>) and all analyzed data supporting this study are provided as supplementary information accompanying this paper.

References

- [1] V. Wagner, A. Dullaart, A.-K. Bock, A. Zweck, *Nat. Biotechnol.* **2006**, *24*, 1211.
- [2] P. Couvreur, C. Vauthier, *Pharm. Res.* **2006**, *23*, 1417.
- [3] D. Bobo, K. J. Robinson, J. Islam, K. J. Thurecht, S. R. Corrie, *Pharm. Res.* **2016**, *33*, 2373.
- [4] K. Letchford, *Eur. J. Pharm. Biopharm.* **2007**, *65*, 259.
- [5] A. Rösler, G. W. . Vandermeulen, H.-A. Klok, *Adv. Drug Deliv. Rev.* **2001**, *53*, 95.
- [6] C. Vauthier, K. Bouchemal, *Pharm. Res.* **2009**, *26*, 1025.
- [7] T. Ehtezazi, N. M. Dempster, G. D. Martin, S. D. Hoath, I. M. Hutchings, *J. Pharm. Sci.* **2014**, *103*, 3733.
- [8] J. Xie, W. J. Ng, L. Y. Lee, C.-H. Wang, *J. Colloid Interface Sci.* **2008**, *317*, 469.
- [9] Y. Yeo, O. A. Basaran, K. Park, *J. Control. Release* **2003**, *93*, 161.
- [10] S. Umez, T. Kitajima, H. Ohmori, Y. Ito, *Sensors Actuators A Phys.* **2011**, *166*, 251.
- [11] M. J. Heller, *Annu. Rev. Biomed. Eng.* **2002**, *4*, 129.
- [12] S. Venkatasubbarao, *Trends Biotechnol.* **2004**, *22*, 630.
- [13] B. H. Lee, T. Nagamune, *Biotechnol. Bioprocess Eng.* **2004**, *9*, 69.
- [14] A. Mant, G. Tourniaire, J. J. Diaz-Mochon, T. J. Elliott, A. P. Williams, M. Bradley, *Biomaterials* **2006**, *27*, 5299.
- [15] S. Hauschild, U. Lipprandt, A. Rumpelcker, U. Borchert, A. Rank, R. Schubert, S. Förster, *Small* **2005**, *1*, 1177.
- [16] W. S. Cheow, T. Y. Kiew, K. Hadinoto, *Eur. J. Pharm. Biopharm.* **2015**, *96*, 314.
- [17] H.-Y. Hsu, S. Toth, G. J. Simpson, M. T. Harris, *AIChE J.* **2015**, *61*, 4502.
- [18] V. Taresco, J. Suksiriworapong, I. D. Styliari, R. H. Argent, S. M. E. Swainson, J. Booth, E. Turpin, C. A. Laughton, J. C. Burley, C. Alexander, M. C. Garnett, *RSC Adv.* **2016**, *6*, 109401.
- [19] D. Kakde, V. Taresco, K. K. Bansal, E. P. Magennis, S. M. Howdle, G. Mantovani, D. J. Irvine, C. Alexander, *J. Mater. Chem. B* **2016**, *4*, 7119.
- [20] M. Giardiello, N. J. Liptrott, T. O. McDonald, D. Moss, M. Siccardi, P. Martin, D. Smith, R. Gurjar, S. P. Rannard, A. Owen, *Nat. Commun.* **2016**, *7*, 13184.
- [21] H. Zhang, D. Wang, R. Butler, N. L. Campbell, J. Long, B. Tan, D. J. Duncalf, A. J. Foster, A. Hopkinson, D. Taylor, D. Angus, A. I. Cooper, S. P. Rannard, *Nat. Nanotechnol.* **2008**, *3*, 506.
- [22] Z.-Y. He, K. Shi, Y.-Q. Wei, Z.-Y. Qian, *Curr. Drug Metab.* **2016**, *17*, 168.
- [23] A. Rosler, G. W. M. Vandermeulen, H. A. Klok, *Adv. Drug Deliv. Rev.* **2001**, *53*, 95.
- [24] N. Kumar, M. N. V. Ravikumar, A. J. Domb, *Adv. Drug Deliv. Rev.* **2001**, *53*, 23.
- [25] A. Endrizzi, Y. Pagot, A. Le Clainche, J. M. Nicaud, J. M. Belin, *Crit. Rev. Biotechnol.* **1996**, *16*, 301.
- [26] C. Romero-Guido, I. Belo, T. M. N. Ta, L. Cao-Hoang, M. Alchihab, N. Gomes, P. Thonart, J. A. Teixeira, J. Destain, Y. Waché, *Appl. Microbiol. Biotechnol.* **2011**, *89*, 535.
- [27] K. K. Bansal, D. Kakde, L. Purdie, D. J. Irvine, S. M. Howdle, G. Mantovani, C. Alexander, *Polym. Chem.* **2015**, *6*, 7196.
- [28] M. Gou, K. Men, H. Shi, M. Xiang, J. Zhang, J. Song, J. Long, Y. Wan, F. Luo, X. Zhao, Z. Qian, *Nanoscale* **2011**, *3*, 1558.
- [29] S. Galindo-Rodriguez, E. Allemann, H. Fessi, E. Doelker, *Pharm. Res.* **2004**, *21*, 1428.
- [30] V. Taresco, R. G. Creasey, J. Kennon, G. Mantovani, C. Alexander, J. C. Burley, M. C. Garnett, *Polymer (Guildf)*. **2016**, *89*, 41.
- [31] P. Kallinteri, S. Higgins, G. a. Hutcheon, C. B. St. Pourain, M. C. Garnett, *Biomacromolecules* **2005**, *6*, 1885.
- [32] V. M. Weiss, T. Naolou, G. Hause, J. Kuntsche, J. Kressler, K. Mäder, *J. Control.*

Release **2012**, 158, 156.

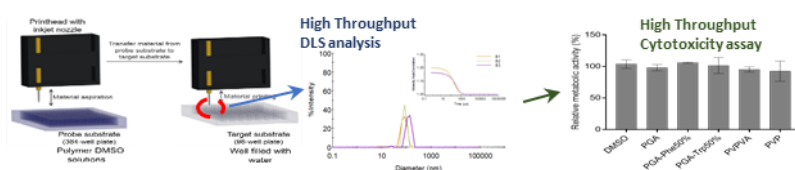
- [33] R. F. Domingos, M. A. Baalousha, Y. Ju-Nam, M. M. Reid, N. Tufenkji, J. R. Lead, G. G. Leppard, K. J. Wilkinson, *Environ. Sci. Technol.* **2009**, 43, 7277.
- [34] K. J. Frank, U. Westedt, K. M. Rosenblatt, P. Hölig, J. Rosenberg, M. Mägerlein, G. Fricker, M. Brandl, *J. Pharm. Sci.* **2014**, 103, 1779.
- [35] A. Dan, I. Chakraborty, S. Ghosh, S. P. Moulik, *Langmuir* **2007**, 23, 7531.
- [36] G. Da Violante, N. Zerrouk, I. Richard, G. Provot, J. C. Chaumeil, P. Arnaud, *Biol. Pharm. Bull.* **2002**, 25, 1600.
- [37] V. M. Weiss, T. Naolou, T. Groth, J. Kressler, K. Mäder, *J. Appl. Biomater. Funct. Mater.* **2012**, 10, 163.
- [38] R. J. Bastin, M. J. Bowker, B. J. Slater, *Org. Process Res. Dev.* **2000**, 4, 427.
- [39] V. Taresco, I. Louzao, D. Scurr, J. Booth, K. Treacher, J. McCabe, E. Turpin, C. A. Laughton, C. Alexander, J. C. Burley, M. C. Garnett, *Mol. Pharm.* **2017**, 14, 2079.
- [40] S. Balbach, C. Korn, *Int. J. Pharm.* **2004**, 275, 1.
- [41] C. T. Rueden, J. Schindelin, M. C. Hiner, B. E. DeZonia, A. E. Walter, E. T. Arena, K. W. Eliceiri, *BMC Bioinformatics* **2017**, 18, 529.

Ink-jet printing is employed as high throughput and miniaturized method to screen nanoparticles (NPs) formulations. Polymer solutions are swiftly dispensed into well-plates using two to three orders of magnitude less material than conventional state-of-the-art techniques. The formation of NPs, their sizes and their cytocompatibility are directly assessed in a single well-plate format, minimizing human-errors and batch variability, and providing pilot data to assist nanoparticle selection and manufacture.

Keyword: self-assembling, ink-jet printer, high throughput-miniaturized screening, Nanoparticles

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High throughput miniaturized screening of nanoparticle formation via inkjet printing





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